

15/10/15

MEASUREMENT OF MELANOCORTIN PEPTIDES AND USES THEREOF**TECHNICAL FIELD**

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The present invention relates to melanocortin peptides and to methods that utilise melanocortin peptides, their measurement, their receptors and biological response systems for the risk assessment and diagnosis of disease. The biological response systems are also utilised to
10 screen for compounds that act as agonists or antagonists of melanocortin receptors.

BACKGROUND

Obesity and type 2 diabetes are major health problems worldwide and are a major threat to health and well-being. Over the last few years
15 significant advances have been made with respect to the molecular determinants of energy balance and insulin resistance. Critical elements of this control system are hormones secreted in proportion to body fat, including leptin and insulin, and their central nervous system targets such as neuropeptide Y and the hypothalamic melanocortin system. Recently
20 proopiomelanocortin and MC4-R have been identified as important targets mediating leptin's activities in the hypothalamus.

Pro-opiomelanocortin (POMC), produced in the pituitary and brain and to a lesser extent in numerous peripheral tissues including skin, pancreas and testis, is the large precursor protein from which melanocortin
25 peptides α -melanocyte stimulating hormone (MSH) and adrenocorticotropin (ACTH) and fragments thereof, are derived. The products of POMC undergo a series of complex, tissue specific, processing events such as further proteolytic cleavages, phosphorylation, α -amidation and NH_2 -terminal acetylation which influence their biological activities. $\text{ACTH}_{1-13}\text{NH}_2$
30 exists as α -MSH and desacetyl- α -MSH. α -MSH, which is acetylated at the N-terminus and amidated at the COOH terminus, is a post translationally modified derivative of $\text{ACTH}_{1-13}\text{NH}_2$ (desacetyl- α -MSH). The acetylation

reaction to form α -MSH is associated with the secretory process; its highest activity is present in the pituitary gland and certain brain regions.

The functional significance of N-terminal acetylation of ACTH₁₋₁₃ in the central nervous system is unknown. N-terminal acetylation of
5 desacetyl- α -MSH to form α -MSH enhances some activities of ACTH₁₋₁₃ and virtually eliminates others. α -MSH injected daily to rats is 10 -100 fold more effective than desacetyl- α -MSH at increasing pigmentation, arousal, memory, attention, and excessive grooming. Desacetyl- α -MSH, however, is more effective than α -MSH at blocking opiate analgesia and opiate
10 receptor binding *in vivo*. α -MSH and desacetyl- α -MSH also differentially affect feeding and weight gain. Weight gain of *agouti* obese mice is increased by subcutaneously administered desacetyl- α -MSH, as is food intake and fat pad weight, but α -MSH injections do not significantly increase food intake or body weight.

15 Despite advances in the understanding of energy homeostasis, efforts have not yielded clinically applicable parameters with which to predict or diagnose pathological imbalances that lead to obesity. There is a need therefore for methods which would assist in the analysis and monitoring of energy metabolism, feeding and weight gain patterns and
20 diagnosis and/or prognosis of associated disorders and diseases.

It is an object of the present invention to ameliorate at least some of the disadvantages of the prior art methods, or at least provide useful alternatives.

25 SUMMARY OF THE INVENTION

According to a first aspect there is provided a method for assessing feeding and/or weight gain pattern in a subject comprising the measurement of a melanocortin peptide in a sample obtained from said subject and comparison of the measured value with a reference value.

30 According to a second aspect there is provided a method for predicting risk of obesity in a subject comprising the measurement of a

melanocortin peptide in a sample obtained from said subject and comparison of the measured value with a reference value

According to a third aspect there is provided a method for diagnosing imbalance in energy homeostasis in a subject comprising the measurement
5 of a melanocortin peptide in a sample obtained from said subject and comparison of the measured value with a reference value.

According to a fourth aspect there is provided a method for diagnosing obesity in a subject comprising the measurement of a melanocortin peptide in a sample obtained from said subject and
10 comparison of the measured value with a reference value

According to a fifth aspect there is provided a method for screening medicaments for the adverse reactions of imbalance in energy homeostasis, feeding/weight gain patterns or obesity in a subject to whom the medicament has been administered comprising the measurement of a
15 melanocortin peptide in a sample obtained from said subject, and comparison of the measured value with a reference value.

According to a sixth aspect there is provided a method for screening foods and/or diets for the adverse reactions of imbalance in energy homeostasis, feeding/weight gain patterns or obesity in a subject to whom
20 the medicament has been administered comprising the measurement of a melanocortin peptide in a sample obtained from said subject, and comparison of the measured value with a reference value. Preferably, the melanocortin peptide measured is either α -MSH or desacetyl- α -MSH.

Preferably the melanocortin peptide measured is α -MSH or
25 desacetyl- α -MSH.

According to a seventh aspect there is provided a method for assessing feeding and/or weight gain pattern in a subject comprising the measurement of at least two melanocortin peptides in a sample obtained from said subject, the calculation of the ratio of the measured melanocortin
30 peptides and comparison of the value of the ratio with a reference value.

According to an eighth aspect there is provided a method for predicting risk of obesity in a subject comprising the measurement of at

least two melanocortin peptides in a sample obtained from said subject, the calculation of the ratio of the measured melanocortin peptides and comparison of the value of the ratio with a reference value.

According to a ninth aspect there is provided a method for
5 diagnosing obesity in a subject comprising the measurement of at least two melanocortin peptides in a sample obtained from said subject, the calculation of the ratio of the measured melanocortin peptides and comparison of the value of the ratio with a reference value.

According to a tenth aspect there is provided a method for
10 diagnosing imbalance in energy homeostasis in a subject comprising the measurement of at least two melanocortin peptides in a sample obtained from said subject, the calculation of the ratio of the measured melanocortin peptides and comparison of the value of the ratio with a reference value.

According to an eleventh aspect there is provided a method for
15 screening medicaments for the adverse reactions of imbalance in energy homeostasis, feeding/weight gain patterns or obesity in a subject to whom the medicament has been administered comprising the measurement of at least 2 melanocortin peptides in a sample obtained from said subject, the calculation of the ratio of the measured melanocortin peptides, and
20 comparison of the value of the ratio with a reference value.

According to a twelfth aspect there is provided a method for screening foods and/or diets for the adverse reactions of imbalance in energy homeostasis, feeding/weight gain patterns or obesity in a subject to whom the medicament has been administered comprising the
25 measurement of at least 2 melanocortin peptides in a sample obtained from said subject, the calculation of the ratio of the measured melanocortin peptides, and comparison of the value of the ratio with a reference value.

Preferably the melanocortin peptide ratio calculated is the ratio of desacetyl- α -MSH to α -MSH.

30 It will be understood that the melanocortin peptides can also be measured by a biological response system in which the resulting profile of response parameters is predictive of the risk of developing obesity or

diagnostic of obesity, imbalance in energy homeostasis or disturbance in feeding/weight gain patterns.

According to a thirteenth aspect there is provided a method of assessing risk of developing obesity, diagnosing obesity or diagnosing an imbalance
5 in energy homeostasis or disturbance in feeding/weight gain patterns in a subject, comprising:

- a. measuring the amount of α -MSH and desacetyl- α -MSH in a sample obtained from the subject, either directly or by subtraction of one of the amount of α -MSH or desacetyl- α -MSH from a measured amount of total
10 MSH in the sample,
- b. calculating the ratio of the amounts of desacetyl- α -MSH to α -MSH.
- c. comparing the ratio of desacetyl- α -MSH to α -MSH with a reference ratio.

The methods of the present invention may utilise quantitative
15 measurements of melanocortin peptides and may do so on intact samples or after separation of melanocortin peptides, in particular desacetyl- α -MSH and α -MSH. Preferably, the separation procedure is selected from chromatography, electrophoresis, immunocapture, affinity capture including receptor-ligand capture or other affinity capture, and the like. It is also preferable that the
20 quantitation procedure is selected from immunoassay including RIA, ELISA, Western blot, immunoprecipitation, and affinity capture, including receptor-ligand capture, peptide-nucleotide affinity capture or other affinity capture, and catalytic reaction-based assay, and the like. More preferably, the separation of the melanocortin peptide is by chromatography and the
25 quantitation is performed by an immunoassay. The chromatographic method described herein, only as an example of such a procedure, is HPLC and the exemplary immunoassay described is RIA. All these detection, quantitation and separation techniques are described in detail in standard laboratory manuals which will be known to those skilled in the art.

30 According to a fourteenth aspect there is provided a method of monitoring treatment for obesity or for imbalance in energy homeostasis and/or disturbance in feeding/weight gain pattern in a subject comprising

contacting a sample obtained from the subject having such treatment with a biological response system wherein the resulting profile of response parameters is indicative of the effect of such treatment on obesity or imbalance in energy homeostasis and/or disturbance in feeding/weight gain pattern.

According to a fifteenth aspect there is provided a method of assessing the risk of developing obesity or developing and/or having an imbalance in energy homeostasis and/or disturbance in feeding/weight gain pattern in a subject comprising analysing the profile of response parameters in a sample from a test subject by comparing it with

- (i) the profile of a sample from a normal subject and
- (ii) the profile of a sample from an obese subject or a subject with an imbalance in energy homeostasis and/or disturbance in feeding/weight gain pattern,

wherein resemblance of the profile of the sample obtained from the test subject to that of the profile in (ii) above, is indicative of that subject being at risk of developing obesity or developing and/or having an imbalance in energy homeostasis and/or disturbance in feeding/weight gain pattern.

Preferably the subject is a mammal and even more preferred is a human subject. Levels of melanocortin receptors (eg. α -MSH and/or desacetyl- α -MSH) may vary with age and between gender. Therefore it is appropriate to compare quantitative levels, ratios and/or biological response parameters in test subjects with those for appropriately sex and age matched control subjects. Of course internal control values may also be used, particularly if monitoring effects of certain drugs or foods, or if monitoring effects of treatments as described herein.

According to a sixteenth aspect there is provided a method of determining the melanocortin peptide status of a sample comprising contacting the sample with a biological response system wherein the resulting profile of response parameters produced by the biological response system indicates the melanocortin peptide status of the sample.

Preferably the sample is a biological fluid such as for example whole blood, plasma, serum, saliva, sweat, urine, amniotic fluid, cord blood, cerebrospinal fluid and the like. The sample may also consist of tissue culture fluid or other medium in case where use is made of cells or tissues

5 In vitro as biological response systems.

According to a seventeenth aspect there is provided a method of screening for a compound which acts as agonist or antagonist of a melanocortin receptor comprising treating a biological response system with a test compound and measuring the resulting profile of response
10 parameters that are indicative of agonist or antagonist activity to the melanocortin receptor.

According to an eighteenth aspect there is provided a method of screening for a compound that is useful in the treatment of obesity comprising exposing a biological response system to a test compound and
15 measuring the resulting profile of response parameters that are indicative of the desired response for the treatment of obesity.

According to a nineteenth aspect there is provided a method of screening for a compound that is useful in the treatment of an imbalance in energy homeostasis or a disturbance in feeding/weight gain patterns comprising exposing a biological
20 response system to a test compound and measuring the resulting profile of response parameters that are indicative of the desired response for the treatment of an imbalance in energy homeostasis or a disturbance in feeding/weight gain patterns.

Preferably, the biological response system is an *in vitro* cell or organ
25 sample or culture capable of responding to melanocortin peptides. The preferred *in vitro* cells are cultures of primary rat osteoblasts, or the UMR106.06 rat osteosarcoma cell line, or the GT1-7 mouse hypothalamic cell line. Any cell line or primary culture of cells that expresses melanocortin receptors, or any combination of such cell lines, may also be
30 used as an *in vitro* biological response system. Some of these cell lines are 3T3-L1 adipocytes, melanocytes, L6 myocytes, B16 melanoma cells, and anterior pituitary cell cultures. Any cell line or primary culture of cells that express melanocortin receptors, or any combination of such cell lines, that

are capable of producing a differential response that distinguishes obese individuals, or individuals at risk of developing obesity, or individuals suffering from an imbalance in energy homeostasis or disturbance in feeding/weight gain patterns, from normal individuals may be used as an *in vitro* biological response system. As the given list is not exhaustive of cell lines or primary cell cultures that express melanocortin receptors, the *in vitro* biological response system described herein is not limited to the use of these. The biological response system may also be an *in vivo* system. Examples of *in vivo* systems include the hypothalamus of a mammal and/or other tissue(s) that are capable of responding to melanocortin peptides.

Of course, it will be understood that a whole animal may be used as an *in vivo* biological response system. In the case where a whole animal is used as an *in vivo* biological response system the response parameters may be feeding frequency and/or body weight gain. Further, samples may be introduced in to the animal biological response system, and tissues and/or organ samples may be obtained from the animal biological response system, which samples may be analysed for the relevant response parameters.

The preferred response profile or fingerprint is one or more proteins or cellular events which differentiate between normal individuals and those at risk of developing obesity, or those suffering from obesity, or those with an imbalance in energy homeostasis, or disturbance in feeding/weight gain patterns.

The preferred response parameters are proteins expressed by the biological response system. Proteins expressed by the biological response system includes but are not limited to stress proteins such as heat shock protein homologue, enzymes such as glyceraldehyde-3-phosphate-dehydrogenase, aldo-keto reductase, citrate synthase, creatine kinase, pyruvate synthase alpha-chain, f1 ATPase beta-chain, and cytoskeletal proteins such as tubulin beta-chain. Other proteins which may be used as response parameters include but are not limited to proteins involved in the melanocortin peptidergic axis, proteins involved in signalling pathways,

enzymes, and membrane-bound proteins. Extracellular effector molecules may also be suitable response parameters.

BRIEF DESCRIPTION OF THE FIGURES.

- 5 **Figure 1.** *Displacement of 125 I- α -MSH bound to rabbit antiserum (1:9000) by increasing amounts of melanocortin peptides.*
Insert: HPLC separation of α -MSH and desacetyl- α -MSH peptides.
- 10 **Figure 2.** *Alpha-MSH but not desacetyl- α -MSH administered i.c.v. significantly decreased food intake.* Food intake was measured over 3h following lateral ventricle injections of vehicle (PBS), 10 μ g α -MSH, or 10 μ g desacetyl- α -MSH to food deprived Wistar rats. (PBS, n = 9; α -MSH, n = 7; desacetyl- α -MSH, n = 10). \square Alpha-MSH significantly decreased food intake to 70% of PBS treated control (*, significantly different from PBS, p < 0.05, one-way ANOVA).
15 Desacetyl- α -MSH has no significant effect on feeding, but there was a trend for a reduction in food intake.
- 20 **Figure 3.** *A higher dose of desacetyl- α -MSH compared to α -MSH administered i.c.v. significantly decreased food intake.* Food intake was measured over 3h following lateral ventricle injections of vehicle (PBS), 10 μ g α -MSH, or 50 μ g desacetyl- α -MSH to food deprived Wistar rats. (PBS, n = 11; α -MSH, n = 11; desacetyl- α -MSH, n=11). (*, significantly different from PBS, p < 0.05, one way ANOVA).
- 25 **Figure 4.** *Desacetyl- α -MSH significantly slowed body weight change in neonatal rats.* Neonatal rats were injected subcutaneously with PBS (n=36), α -MSH (n=27) or desacetyl- α -MSH (n=27) (0.3 μ g/g body weight/day) for their first 14 days of life. There were no significant differences in body

weight over 14 days between PBS and α -MSH treated pups. Neonatal rats treated with desacetyl- α -MSH for 14 days grew significantly slower than either PBS or α -MSH treated pups ($p < 0.05$, GLM repeated measures analysis of variance, SAS system).

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Figure 5 *RT-PCR shows MC2-R, MC4-R and MC5-R expression in primary rat osteoblast cells.* Lane 2, MC2-R PCR product (290p); lane 4, MC4-R PCR product (554bp); lane 6, MC5-R PCR product (290bp); controls of specificity were the absence of RT in the reverse transcription reaction mixture (lane 3, MC2-R; lane 5, MC4-R; lane 7, MC5-R). The primers used are shown in Table 1. The PCR products were run on a 2% agarose gel alongside a HindII-EcoRI digested lambda DNA molecular weight marker (lane 1).

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Figure 6 *Northern blot analysis showed MC4-R mRNA transcripts in primary rat osteoblasts.*

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Poly (A⁺) mRNA (5 μ g) from rat brain (lane 1) and primary rat osteoblasts (lane 2) were separated by formaldehyde-agarose gel electrophoresis (1.2%), transferred to a nylon membrane and probed with a ³²P labeled specific rat MC4-R DNA fragment. A digital image was obtained with a Storm imaging system screen and scanner. An RNA ladder was run on the gel and used to determine the mRNA sizes (2.0 – 2.6).

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Figure 7 *Ribonuclease Protection Assay shows MC4-R mRNA expression in UMR106.06 and primary rat osteoblast cells.* Lane 2, full length rat MC4-R riboprobe (562bp), probe incubated with: lane 3, 1 μ g/ml RNase A and 50 U RNase T1; lane 4, 10 μ g tRNA; lane 5, 10 μ g rat brain poly (A⁺) mRNA, lane 6, 10 μ g primary rat osteoblast poly (A⁺) mRNA; lane 7, 10 μ g UMR106.06 poly (A⁺) mRNA. The labeled fragments were run on a 6% polyacrylamide gel alongside a radiolabeled

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123bp DNA Ladder (GIBCO BRL) (lane 1). The data shown are representative of three independent experiments.

Figure 8. *Alpha-MSH stimulation of rat primary osteoblast proliferation.*

5 Growth arrested primary rat osteoblasts were stimulated with increasing doses of α -MSH and [3 H] thymidine uptake (a) and changes in cell number (b) measured. Data are expressed as mean \pm SEM. Significant difference from control; * = $p < 0.04$, ** $p < 0.001$

Figure 9. *Desacetyl- α -MSH and ACTH1-24 antagonise α -MSH*

10 *stimulated stimulation of thymidine incorporation into cultures of rat primary osteoblasts.* Growth arrested primary rat osteoblasts were stimulated with either 10^{-7} M or 10^{-8} M α -MSH alone (a,b), 10^{-7} M desacetyl- α -MSH alone (a), ACTH₁₋₂₄ alone (b), or combinations of α -MSH and desacetyl- α -MSH (a) or α -MSH and ACTH₁₋₂₄ (b) and [3 H] thymidine uptake measured. Data are expressed as mean \pm SEM. Significant difference from control; * = $p < 0.04$, ** $p < 0.001$

Figure 10. *Bi-phasic Dose response curve for treatment of*

20 *UMR106.06 with alpha-MSH.* UMR106.06 rat osteosarcoma cells were stimulated with 10^{-8} to 10^{-12} alpha-MSH and the [3 H] thymidine uptake measured.

Figure 11. *Dose response curve for treatment of UMR106.06 with*

25 *desacetyl-alpha-MSH.* UMR106.06 rat osteosarcoma cells were stimulated with 10^{-8} to 10^{-12} desacetyl-alpha-MSH and the [3 H] thymidine uptake measured.

~~{Figure 12A to C. *Proteome analysis.* Figures 11 A to C show differences in protein profiles after treatment with alpha-MSH and desacetyl-alpha-MSH.}~~

Figure 12 [43] *Effects of alpha-MSH on Thymidine incorporation*

30 *in Chondrocyte monolayers.* The figure shows increased thymidine incorporation (interpreted as increased cell proliferation) in response to stimulation by alpha-MSH.

DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention is based on a surprising observation that the balance/abundance/status of MSH peptides in the circulation, may correlate with, and be predictive of, the development of an imbalance in energy homeostasis, disturbance in feeding/weight gain patterns and ultimately obesity.

Just as the measurement of "good" (HDL) and "bad" (LDL) cholesterol predicts cardiovascular risk, we have discovered that the balance, i.e. the ratio, of melanocortin peptides α -MSH and desacetyl- α -MSH is particularly predictive and/or diagnostic of imbalances in energy homeostasis, disturbances in feeding/weight gain patterns and ultimately obesity. However, absolute level of individual, or combination of, MSH peptides will also serve this purpose.

A novel approach described herein involves the use of a biological response system that processes stimulus through melanocortin receptors, and which outputs information through various response parameters. Of course, simple quantitative measurement of MSH peptides in samples of biological fluids, such as antibody-based methods, and the use of such data to determine ratios of MSH peptides, may also be used in the prognostic/diagnostic methods of the present invention. The biological response system may be used in conjunction with the simple quantitative measurements, to enhance the power of the methods described herein.

The measurement of specific MSH peptides in subject's plasma or other biological fluids, as described herein in one embodiment, follows extraction and fractionation using high pressure liquid chromatography (HPLC), followed by classical RIA, according to modified methods described in the literature (Facchinetti, F., Bernasconi, S., Iughetti, L., Genazzani, A.D., Ghizzoni, L., Genazzani, A.R. Changes in dopaminergic control of circulating melanocyte-stimulating hormone-related peptides at puberty. *Pediatric Research* 38; 91-94, 1995; Mauri, A., Volpe, A., Martellotta, M.C., Barra, V., Piu, U., Angioni, G., Angioni, S., Angiolas, A. α -Melanocyte-stimulating hormone during human perinatal life. *J Clin*

Endocrinol Metab 77: 113-117, 1993; Mauri, A., Martellotta, M.C., Melis, M.R., Caminiti, F., Serri, F., Fratta, W. Plasma alpha-melanocyte-stimulating hormone during the menstrual cycle in women. Hormone Research 34: 66-70, 1990). This approach was adopted initially to verify
5 the identity of the MSH peptides and ascertain the functionality of the immuno-based and biological response methodology. Simple quantitative immuno-assay type methods for measuring MSH peptides in a sample can be employed with equivalent results.

Analysis of the abundance of and, particularly the ratios of, α -MSH
10 and desacetyl- α -MSH in blood circulation or other body fluid containing MSH peptides, are novel developments in the field of prediction and/or diagnosis of predisposition to obesity.

For the purposes of the invention herein described, the term
"biological response system" includes any whole animal, organ, tissue or
15 cell which is able to respond to a melanocortin peptide or an effector molecule generated by a response to a melanocortin peptide.

For the purposes of the invention herein described, the term
"response parameter" includes a cellular product (which may be a protein,
nucleic acid, lipid, carbohydrate or a combination of these), or a
20 measurable cellular event, resulting from interaction of the biological response system with a melanocortin peptide, for example cell proliferation, cell cycle progression, cell differentiation and the like, mass spectrometry or currently commercially available gene expression arrays may be used to monitor these response parameters, among other techniques.

25 Not wishing to be bound by any particular theory, when the biological response system is treated with melanocortin peptides, or a sample containing melanocortin peptides, the profile or "fingerprint" of response parameters resulting from melanocortin receptor stimulus also reflects the melanocortin peptide balance/abundance/status of the sample. A
30 comparison of the fingerprints of response parameters resulting from normal subjects and obese individuals, or individuals with an imbalance in energy homeostasis and/or disturbance in feeding/weight gain patterns

provides additional information, by way of profile databases, that may be used to predict imbalance in energy homeostasis and/or disturbance in feeding/weight gain patterns or the risk of onset of obesity or that may be diagnostic of these conditions.

5 For the purpose of the invention described herein, the term "profile" or "fingerprint of response parameters" is a reference to one or a plurality of response parameters that may be ascertained by various techniques, which are indicative of an imbalance in energy homeostasis and/or disturbance in feeding/weight gain patterns, obesity or the risk of onset of obesity.

10 The response parameters that are profiled in the biological response systems may be the result of a primary response by the system to stimulus by melanocortin peptides, or they may be the result of a secondary response following the primary response to melanocortin peptides. The response profile may be utilised to monitor treatments used for obesity.

15 The profiles may also be used to monitor the onset of obesity [, the efficacy of treatment, relapse or progression of or imbalance in energy homeostasis and/or disturbance in feeding/weight gain patterns. The profile of parameters may therefore be adopted as a clinician's tool to assess risk of developing disease, diagnose disease, monitor disease and
20 monitor treatment of disease.

 The biological response system is also useful to screen for compounds that are effective in the treatment of imbalances in energy homeostasis and/or disturbances in feeding/weight gain patterns or obesity. The system would also be useful to screen for compounds that act as
25 agonists or antagonists of melanocortin receptors. The response to test compounds, reflected in the resulting profile of response parameters, may be monitored by mass spectrometry or currently commercially available gene expression arrays, among other techniques. Such compounds are potential candidates for the treatment or prevention of obesity, or an
30 imbalance in energy homeostasis, or a disturbance in feeding/weight gain patterns, or other metabolic imbalances brought about by disturbances in

melanocortin peptide balance/abundance/status and the resultant receptor response.

Preferred embodiments of the invention will now be described by way of example only with reference to the following examples.

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EXAMPLES

Example 1. Method for separation and detection/quantitation of α -MSH and desacetyl- α -MSH in plasma extracts

1.1. Extraction of Plasma using sep-Pak C18 Cartridge

Plasma (1-2 mL rodent or 10-20 mL human) was collect on ice and
10 equal volume of 0.1M HCl add, and left for 30 minutes on ice. The plasma was spun for 30 minutes at 3300rpm at 4°C before use.

Sep Pak C18 cartridges (Waters Corporation, MA, USA) were pre-washed with 10mL methanol followed by 10 mL phosphate buffered saline (PBS). Sample was loaded onto column at flow rate of 5-10 mL per
15 minute. 3mL of 10% methanol in 0.5M acetic acid was run over to elute non-specific or interfering substances (5-10 mL per minute). MSH peptides were eluted with 9mL 90% methanol in 0.5M acetic acid into silicnised tubes, then freeze dried to dryness with 900 μ g polypep (Sigma-Aldrich, MO, USA) and 9 μ L of 330 μ M n-octyl- β -D-glucopyranoside (Sigma-Aldrich,
20 MO, USA) added to each tube.

1.2 Separation of α -MSH and desacetyl- α -MSH using HPLC

Freeze dried mixture (after Sep-Pak extraction) was reconstituted in 150 μ L HPLC buffer (acetonitrile: 0.1% trifluoroacetic acid (TFA) mixed at a ratio of 18:82). The sample was spun In Eppendorf tube to remove any
25 precipitated material before transferring the sample to HPLC.

100 μ l of sample was injected onto HPLC C18 column (μ Bondpack, 39 x 300 mm, 10 μ M size) and fractions collected by eluting with a linear gradient from 18-40% acetonitrile in 0.1% TFA at a flow rate of 1.5 mL/min.

Fractions were collected into 6 mL siliconised glass kimble tubes each of which contained 15 μ L of 10 mg/mL polypep and 1.5 μ L of 330 μ M n-octyl- β -D-glucopyranoside (Sigma-Aldrich, MO, USA). The fractions were freeze dried.

- 5 The retention times were: α -MSH, 8.6 minutes, and desacetyl- α -MSH, 6.5 minutes (Figure 1: Insert). It will be appreciated by those skilled in the art that this separation technique is applicable to samples other than plasma extracts. In fact it will be applicable without significant alterations to any biological fluid containing MSH peptides as well as samples of purified
- 10 MSH peptides.

The separated α -MSH and desacetyl- α -MSH peptides are then quantitated using a sensitive and specific Immunoreactive assay.

1.3 Radiolimmunoassay of MSH peptides.

α -MSH and desacetyl- α -MSH were obtained from Bachem AG,

- 15 Hauptstrasse 144, Switzerland

Alpha-MSH

Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH₂
(Bachem # H-1075.0001)

Desacetyl-alpha-MSH

- 20 H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH₂
(Bachem #H-4390.0001)

Freeze dried samples were reconstituted in RIA assay buffer (rodent – 200 μ L; human – 300 μ L). RIA assay buffer: 0.05 M phosphate buffer pH 7.4, 0.1 M NaCl, 0.5% BSA, 10 mM EDTA,

- 25 ¹²⁵I α -MSH was diluted to 10,000 cpm in RIA assay buffer.

α -MSH standards were prepared in RIA assay buffer: 0.00075, 0.001, 0.0015, 0.002, 0.003, 0.004, 0.005, 0.0075, 0.01, 0.015 ng/100 μ L

Desacetyl- α -MSH standards were prepared in RIA assay buffer: 0.001, 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, 0.5 ng/100 μ L

Assay procedure: tubes set up in duplicate with the following:

- a) 100 μ L standard or sample
- b) 100 μ L rabbit polyclonal antibody (KM4), 1:20,000 diluted in RIA assay buffer
- 5 c) Vortex and incubate overnight at 4°C
- d) Add 100 μ L 125 I- α -MSH (10,000 cpm) to each tube
- e) Vortex and incubate overnight at 4°C
- f) Prepare secondary antibody mix: 8% PEG 6000 in 0.01M PBS, 1% #2 sheep anti-rabbit gamma globulin, 0.025% normal rabbit serum.
- 10 g) Add 1mL secondary antibody mix to each tube
- h) Vortex and Incubate 1 hour at room temperature.
- i) Spin at 3300rpm, 4°C for 45 minutes,
- j) Drain off supernatant
- 15 k) Count residue in gamma counter

1.4 Development of polyclonal anti- α -MSH antibody

A high affinity antibody was raised following Immunisation with synthetic α -MSH (N-Acetyl-SYSMEHFRWGKPV-NH₂) (purchased from Bachem, AG, Hauptstrasse 144, CH-4416, Bubendorf, Switzerland) conjugated to Keyhole limpet hemacyanin (KLH) according to conventional procedure described in well known literature (Antibodies. A Laboratory manual. E. Harlow & D. Lane. Cold Spring Harbor Laboratory, 1988) to each of 4 rabbits. A total of 8 injections were given at 3-week intervals. The details are as follows:

- 25 1. Four rabbits were immunised with 150 μ g α -MSH conjugated to 300 μ g KLH with glyceraldehyde per rabbit.
- 2. Immunisations were carried out by Animal Resource unit, University of Auckland. First immunisation used complete Freund's adjuvant. All other immunisations (3 weeks apart) used incomplete Freund's adjuvant.
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3. One rabbit (KM4) developed antibodies that recognised both α -MSH and desacetyl- α -MSH.

1.5 *Lactoperoxidase iodination of α -MSH*

1. Add 5 μ L (2 μ g) α -MSH in water to an Eppendorf tube.
- 5 2. Add 5 μ L Na¹²⁵I (0.5 μ Cl) to the α -MSH in Eppendorf tube.
3. Add 47 μ L 0.1 M Na Acetate buffer, pH 5.6.
4. Add 10 μ L lactoperoxidase (Sigma-Aldrich, MO, USA) freshly diluted in water (2 μ g/100 μ L).
5. Add 5 μ L H₂O₂ freshly diluted 1:7,500 in water.
- 10 6. Mix and incubate 5 minutes at room temperature.
7. Repeat steps 5 & 6 two more times.
8. Stop reaction by adding 500 μ L PBS and 100 μ L transfer buffer (Transfer buffer = RIA Assay buffer with 0.1% Triton X-100 (Sigma-Aldrich, MO, USA) and 0.05% NaN₃).
- 15 9. Load mix onto a G2 chromatography column (Pharmacia K9) and elute with Transfer buffer.
10. Collect 1 mL fractions, count 10 μ L of each fraction in gamma counter to identify the relevant protein peak.
11. Pool the 3-4 tubes on the descending side of the relevant protein peak.
- 20

To test the antisera 5 μ g α -MSH was iodinated and purified. The iodinated material was incubated overnight at 4°C with diluted antiserum and increasing amounts of unlabeled melanocortin peptides. One rabbit developed a high affinity antibody which recognised both α -MSH and desacetyl- α -MSH and not ACTH, γ 1, γ 2, or γ 3-MSH (Figure 1).

Example 2: Plasma MSH peptide content in normal and obese mice

Adult male mice were anaesthetised with halothane and decapitated. Blood was collected into ice cold tubes containing EDTA. The plasma was separated by centrifugation at 4000rpm for 10 minutes at 4°C. Plasma from 3-4 mice was pooled and mixed, extracted using Sep-Paks, and MSH

peptides separated using HPLC and quantitated using RIA. Table 1 below shows the MSH data.

Table 1: Plasma from 3-4 mice were pooled and assayed for MSH peptides using HPLC and RIA assays.

MOUSE TYPE	α -MSH (pg/ml)	des- α -MSH (pg/ml)	α -MSH + des- α -MSH (pg/ml)	des- α -MSH/ α -MSH
obese				
lean				

5

The obese mice had a substantially higher des- α -MSH/ α -MSH ratio than the lean mice. This was primarily due to a substantially lower level of α -MSH in the obese animals. Within a population this can also be interpreted as having high des- α -MSH in the obese subjects.

10 **Example 3: In vivo biological response of the hypothalamus to alpha-MSH and desacetyl-alpha-MSH peptides.**

Alpha-MSH and desacetyl- α -MSH both couple melanocortin receptors to either adenylyl cyclase or calcium-signalling pathways *in vitro*. To characterise the signal transduction pathways engaged by α -MSH and desacetyl- α -MSH *in vivo*, rats received an intracerebroventricular (i.c.v.) injection of either phosphate buffered saline (PBS), α -MSH or desacetyl- α -MSH. Three hours later, food intake was measured and hypothalamic tissues were collected for 2D gel electrophoresis-based proteome analysis. ***Intracerebroventricular Injection of melanocortin peptides in adult rats.***

20

Animals:

Adult male Wistar rats (50-60 days old, 230-260g at the beginning of the experiment) were maintained in individual cages under controlled temperature (23°C) and reverse lighting (1000-2200 lights off). Standard laboratory chow (NZ Stockfeed Ltd) and tap water were available *ad libitum* during the adaptation phase. During this time animals were handled daily to minimize the effects of stress on food intake during experiments. Body weight was measured daily before, and one week after cannulation. Any

25

animal showing signs of illness, such as weight loss, poor grooming, or decreased activity, was removed from the study. All animal procedures undertaken were approved by the Animal Ethics Committee of the University of Auckland.

5 Cannula placement:

After 7 days of adaptation, animals were subject to cannula placement surgery under 3% halothane /O₂ anaesthesia. A permanent lateral ventricle infusion cannula (6-mm 21 gauge) was placed on top of the dura at 7.5 mm anterior from stereotaxic zero, 1.5 mm to the right of the mid-sagittal line, and secured to the skull with dental cement. Animals were
10 allowed at least 7 days to recover from surgery before injections.

ICV Infusion of melanocortin peptides.

Rats were fasted overnight before the day of experiment. Starvation serves to increase baseline food intake during the initial few hours of testing
15 melanocortin peptide effects on food intake, thereby providing a greater range in which the effect of the anorectic agent α -MSH could be demonstrated.

Under 3% halothane /O₂ anaesthesia rats were infused icv through a 12-mm 27-gauge needle, connected to 20-cm length tubing attached to a
20 syringe. Infusions were performed in the early dark phase between 1000 and 1130 hr using motor driven infusion pumps at a rate of 1.0 μ l/min over 10 min. Movement of a 0.2 ml air-space introduced between the 0.9% saline solution filling the PE10 tubing-syringe system and the test solution served as an indicator of a successful infusion. At the end of each
25 experiment animals were euthanised by pentobarbital overdose, and cannula placement was confirmed by visual inspection of the cannula tip location within the brain ventricular system.

Proteome analysis

Proteome analysis showed that the expression of 14 proteins were
30 significantly different between PBS and α -MSH, and 20 proteins were significantly different between PBS and desacetyl- α -MSH treated groups ($p < 0.05$, non-parametric/Mann-Whitney U test). Only one of these proteins

was common to α -MSH and desacetyl- α -MSH. A combination of Reverse-phase HPLC followed by Edman protein sequencing, and peptide mass fingerprinting technique using MALDI-TOF mass spectrometry were used to identify the proteins of interest. The proteomic data provide a snap-shot of the protein expression patterns in the hypothalamus 3 hours post i.c.v. administration of the melanocortin peptides. The expression of different hypothalamic proteins following administration of either α -MSH or desacetyl- α -MSH supports the hypothesis that these peptides activate different biological responses *in vivo* by activating different molecular and cellular signalling pathways (Figures 11 A to 11C).

Tables 1 and 2 represent data from central injection of MSH peptides into brain.

Tables 3 and 4 represent data from a neonatal study, where the two MSH peptides were injected subcutaneously into new-born rats for 14 days, and the changes in hypothalamic proteins assessed with the same method as the above study.

Tables below also show identity of proteins useful as a profile or as markers for the biological response system.

Table 1

Spot no.	Protein name	Accession no.	Database Mr	Protein coverage	Score	no. datafiles matching
Proteins significantly changed by α-MESH treatment						
p428	Vimentin*	g/2078001	51546	2.2	2.661#	1 (2*)
p540	Heat shock 70kD	g/13435656	70809	11.6	50.3	8
p711	Similar to tubulin beta polypeptide	g/13097483	33883		2.446#	1 (2*)
p1350	Similar to heat shock 71kD*	g/20853631	33053	17.9	26.3	5
	Cofilin 1	g/12861068	24761		3.5803#	1 (2*)
p1528	F1-ATPase beta subunit*	g/2030033	36729	15.4	40.4	4
	GAPDH	g/8393418	35787	8.1	18.5	4
	Tubulin	g/13324579	49477	5	10.3	2
p1625	Diazepam binding inhibitor	g/13837378	10028	13.6	30.3	2
	Alpha-endase	g/20850614	47625	4.2	38.3	3
Proteins significantly changed by desacetyl-α-MESH treatment						
p582	GAPDH*	g/8393418	35787	10.5	30.4	4
	Aldo-keto reductase family 1*	g/13591894	36464	5.5	20.3	3
	Citrate synthase*	g/16543177	51615	8.2	10.3	3
p1267	Phosphoethanolamine binding protein*	g/8393910	20801	25.1	10.5	3
	Malate dehydrogenase	g/15100179	38442	6.6	20.3	2
p1347	Cu/Zn superoxide dismutase	g/1213217	15897	16.1	20	2
p1438	Gammaglutamyl transaminase	g/118348	47092	3.5	20.8	2
p1521	Triosephosphate isomerase*	g/12821074	26886	10.8		2
	Tubulin beta polypeptide	g/13097483			2.581#	1 (2*)
p1548	Pyruvate kinase 3	g/20890302	57885	5.1	40.3	2
	Iso citrate dehydrogenase 3 (NAD+) alpha	g/16250284	39639	6	20.4	2
	Phosphoethanolamine binding protein	g/8393910	20801	15	30.4	2
	GAPDH	g/20845424	35828	6.3	10.4	2
p1687	Acidic type mitochondrial creatine kinase	g/125316	46981	4.8	21.1	2

Table 2

Protein	α -MSH effect compared to control	des- α -MSH effect compared to control
Stress protein		
heat shock protein homologue (p540)	2.3 fold increase	
heat shock protein homologue (p1350)	2.7 fold increase	
Enzymes		
Protein disulfide isomerase (p261)		1.4 fold decrease
glyceraldehyde-3-phosphate-dehydrogenase (p1210)		1.4 fold decrease
creatine kinase (p706)	2.0 fold increase	
triosephosphate isomerase (p1521)		1.4 fold decrease
gamma-enolase (p1438)		1.7 fold decrease
Cu/Zn superoxide dismutase (p1347)		1.4 fold decrease
Cytoskeletal proteins		
tubulin beta chain (p711)	1.6 fold increase	
Vimentin (p428)	1.6 fold increase	
Signaling proteins		
phosphatidylethanolamine binding protein (p1267)		2.5 fold decrease

Table 3

Spot no. Protein name	Accession no.	Database Mr	Matches	Coverage %	Score
p537 dihydropyrimidinase-like 2	gi/2087656 0	62.3	7	16.08	68.4
p1079 creatine kinase, mitochondrial 1	gi/2091154 1	47.0	2	5.52	20.3
p1251 creatine kinase, brain	gi/6978659 gi/1675834	42.7	5	18.37	58.4
p1317 thiol-specific antioxidant protein	8	24.8	10	50.44	96.8
p1332 tubulin beta					
p1339 triosephosphate isomerase	gi/68423	26.7	8	36.15	156.7
ATP synthase, H ⁺ transporting,					
p1351 mitochondrial	gi/6680748	59.8	8	15.55	164.3
p1360 spectrin alpha chain, brain,	gi/1738050				
p1362 fragment	1	28.5			114.3
similar to phosphoglycerate kinase	gi/2084475				
p1363 1, fragment	0	44.6	5	12.95	46.3
ATP synthase, H ⁺ transporting,					
p1379 mitochondrial	gi/6680748 gi/1738925	59.8	4	4.88	60.3
p1381 hypothetical protein	7	25.8	5	20.26	70.3
glial fibrillary acidic protein (GFAP),					2.807
p1414 fragment	gi/387163	46.8	1	2.98	#
p1445 heat shock protein 70kDa, fragment					3.619
p1454 triose-phosphate isomerase	gi/68423	26.7	1	5.6	#
similar to prohibitin (B-cell	gi/2091289				2.726
p1458 receptor), fragment	5	29.8	1	3.68	#
p1488 tubulin alpha3	gi/6678465 gi/1309748	50.0	1	14/?	#
p1520 similar to tubulin beta polypeptide	3	34.0	5	24.83	50.3
ATP synthase, H ⁺ transporting,					
p1532 mitochondrial	gi/6680748	59.8	2	3.25	20.3
p1542 cofilin 2	gi/6671746	18.7	5	34.94	48.4
creatine kinase, brain, fragment	gi/6978659	42.7	3	12.34	28.3
lactate dehydrogenase B, fragment	gi/6881148 gi/2082377	36.6	3	6.89	40.3
p1557 similar to SH3-containing protein	8	44.1	3	7.34	36.3
p1558 tumor necrosis factor	gi/7305585	25.9	1	16/?	#
ATP synthase, H ⁺ transporting,					
p1567 mitochondrial	gi/6680748	59.8	4	4.88	116.3
p1588 stathmin, Ser38*	gi/8393696	17.3	11	47	188.6
p1610 stathmin	gi/8393696	17.3	12	55.7	228.1
spectrin alpha chain, brain,	gi/1738050				
p1690 fragment	1	28.8	8	3.2	114.3
p1754 tubulin	gi/1284675				6.928
p1757 unknown protein	8 gi/1739129	49.6 27.0	1 2	4.1 6.98	# 28.3

25

5

histidine triad nucleotide-binding p1790 protein	gi/2088059 0	13.8	5	42.9	86.7
glyceraldehyde-3-phosphate p1827 dehydrogenase	gi/8393418 gi/1286106	35.8	4	8.11	62.4
p1854 cofilin 1, fragment p1936 creatine kinase, brain	8	24.8	3	8.3	50.3

Table 4

Protein identity	Protein no.	Level compared to control
Proteins changed by α-MSH treatment:		
Metabolic enzymes		
ATP synthase H ⁺ transporting	p1381	2.5 fold increase
ATP synthase H ⁺ transporting	p1667	2.2 fold increase
ATP synthase H ⁺ transporting	p1532	2.0 fold increase
creatine kinase brain	p1079	1.8 fold increase
triosephosphate isomerase	p1454	5.0 fold increase
cytoskeleton		
tubulin alpha	p1468	2.7 fold increase
tubulin beta	p1332	1.4 fold increase
tubulin beta	p1520	2.4 fold increase
tubulin beta	p1754	1.8 fold increase
spectrin fragment	p1690	1.8 fold increase
glial fibrillary acidic protein	p1414	1.4 fold increase
cofilin	p1854	2.5 fold increase
signalling		
prohibitin homologue	p1458	1.7 fold increase
stathmin	p1610	2.1 fold increase
stress response		
thiol-specific antioxidant protein	p1317	4.2 fold increase
heat shock protein	p1445	2.1 fold increase
Unknown function		
protein kinase C inhibitor	p1790	2.0 fold increase
Proteins changed by desacetyl-α-MSH treatment:		
Metabolic enzymes		
creatine kinase brain	p1079	1.9 fold increase
creatine kinase brain	p1251	2.1 fold increase
triosephosphate isomerase	p1339	2.1 fold increase
similar to phosphoglycerate kinase	p1363	1.8 fold increase
ATP synthase, H ⁺ transporting	p1379	1.7 fold decrease
Cytoskeleton		
spectrin fragment	p1382	2.5 fold decrease
cofilin	p1854	1.9 fold increase
tubulin beta	p1620	1.8 fold increase

Signalling			
stathmin		p1610	2.6 fold increase
stathmin	P-Ser38	p1588	1.7 fold increase
prohibitin homologue		p1458	1.7 fold increase
Stress response			
heat shock protein		p1445	2.0 fold increase
dihydropyrimidinase-like 2		p637	2.5 fold decrease
Unknown proteins			
RIKEN cDNA0810011D08		p1381	3.3 fold increase
similar to SH3-containing protein SH3GLB2		p1557	4.0 fold increase
protein kinase C inhibitor		p1790	1.8 fold increase
hypothetical protein XP_112457		p1938	2.2 fold increase

Measurement of food intake:

Following infusion, the cannula was left in place for 1 min, removed, and the animal returned to its cage with fresh pre-weighed food and water.

- 5 At 3 h post-injection, the pellets and collected food spillage in the cage, were weighed and this weight was subtracted from the initial weight to quantify the amount of food eaten over 3 h.

Statistical analysis:

- The significance of treatment effects was evaluated using one-way
10 ANOVA (Systat10 package)

RESULTS***Alpha-MSH is more potent than desacetyl- α -MSH at inhibiting food intake.***

- Alpha-MSH (10 μ g) administered i.c.v to food deprived adult rats just
15 prior to the 12h dark cycle significantly reduced food intake over 3h compared to PBS treated control animals (α -MSH, n = 7; PBS, n = 9; p<0.05). There was a trend for desacetyl- α -MSH (10 μ g) to also decrease food intake (n=10) over 3h, but this was not significantly different from the PBS treated control group of rats.

- 20 A 5-fold higher dose of desacetyl- α -MSH (50 μ g) did significantly reduce food intake over 3h compared to PBS treated control animals (desacetyl- α -MSH, n = 11; PBS, n = 11 p<0.05) in a second independent study. In this study α -MSH (10 μ g) again significantly inhibited food intake over 3h compared to PBS treated control animals (α -MSH, n = 11; p<0.05).

25

Example 4: *In vivo* biological response to the subcutaneous administration of alpha-MSH and desacetyl-alpha-MSH peptides in rats.

- The activity of alpha-MSH and desacetyl-alpha-MSH when
30 administered peripherally was measured by subcutaneous administration to postnatal rats for 14 days.

Animals:

Adult female Wistar rats were housed in plastic cages and kept on a 12-h dark/light cycle. Animals received tap water and rat pellets *ad libitum* and were mated with males of the same strain. Each litter of new-born

5 Wistar rats was culled to 9 pups per mother.

Subcutaneous Injections of melanocortin peptides:

Each litter was assigned to a treatment group; vehicle, phosphate buffered saline (PBS), α -MSH (0.3 μ g/g body weight/day), or desacetyl- α -MSH (0.3 μ g/g body weight/day). PBS or freshly prepared peptide solutions
10 made up freshly in PBS containing 0.1% BSA were injected subcutaneously once per day in a volume of 40 μ l for 14 days. Animals were injected on day 14 and 1h later they were euthanised using sodium pentobarbital.

Measurement of body and organ weights:

Rats were weighed at birth and then every 2 days prior to injection of
15 peptides. Body weights were recorded on day 14 before injection and again when they were euthanised. The following organs were dissected and weighed: brain, heart, kidney, liver, lung, spleen.

Statistical analysis:

Linear relationships between organ weights and body weights was
20 tested using regression analysis of the organ weights measured against final body weight on day 14. There were significant linear relationships between organ weights and body weights for the following tissues: brain, spleen, heart, kidney and liver. There was no significant regression between lung weight and body weight. For those organs where their weight
25 was linearly correlated to body weight, treatment effects on organ weight changes were analysed using ANCOVA with body weight as the co-variate.

Differences in body weight were analysed using a General Linear Model with repeated measures. Significance was assumed at the $P < 0.05$ level.

30 ***Desacetyl- α -MSH significantly slowed body weight change in neonatal rats.***

Three litters of neonatal rats injected daily with desacetyl- α -MSH (0.3 μ g/g body weight/day) for their first two weeks of life grew significantly slower than control pups injected daily with PBS (4 litters). In contrast, α -MSH (0.3 μ g/g body weight/day) injected daily in neonatal rats (3 litters) had no significant effect compared to control pups injected with PBS. Body weight data obtained from these subcutaneous injections of melanocortin peptides were analysed as a nested within nested design, with the following independent factors: Treatment effects, Litter (Treatment) effects, and Rat (Litter * Treatment) effects. This analysis allowed the separation of sources of variation due to treatment effects, from between litter and between individual rat, differences. Data were analysed using a General Linear Model with repeated measures. Pups treated with desacetyl- α -MSH (n=27) grew significantly slower than either vehicle control (n=36) or alpha-MSH treated pups (n=27) (p,0.05, repeated measures analysis of variance, SAS).

Both α -MSH and desacetyl- α -MSH-treated neonatal rats appeared to catch up on body weight from day 12 compared to control PBS treated rats.

Different effects of subcutaneously administered α -MSH and desacetyl- α -MSH on organ weights in neonatal rats.

Both α -MSH and desacetyl- α -MSH (0.3 μ g/g body weight/day) administered subcutaneously daily for 14 days to neonatal rats, significantly decreased brain weight compared with control PBS treated animals. Alpha-MSH significantly decreased kidney weight but desacetyl- α -MSH had no significant effect on kidney weight. Desacetyl- α -MSH, however, significantly increased spleen weight but α -MSH had no significant effect on spleen weight.

Example 5: *In vitro* melanocortin receptor-mediated biological response system

***In vitro* biological response of primary rat osteoblasts and UMR106.06 rat osteosarcoma cells to melanocortin peptides.**

Materials:

The melanocortin peptides, ACTH₁₋₂₄, desacetyl- α -MSH and α -MSH were purchased from Bachem California (CA, USA). The production of recombinant mouse agouti protein has previously been described (Willard, 1995 #760). [³H] Methyl thymidine was purchased from Amersham Life Science (Buckinghamshire, U.K.).

Cells:

Rat osteosarcoma UMR106.06 cells were grown in Dulbecco's modified Eagle's Medium (DMEM) (GIBCO BRL, Rockville, MD) supplemented with 10% fetal calf serum (FCS) (In Vitrogen, Auckland, NZ) and 50 U/ml penicillin plus 50 μ g/ml streptomycin. Cells were maintained at 37°C in 5% CO₂ and passaged every week.

Primary rat osteoblasts were isolated from 20 day fetal rat calvariae. (The use of animals for these studies was approved by the Auckland Animal Ethics Committee.) Calvariae were excised and the frontal and parietal bones, free of suture and periosteal tissue, were collected and sequentially digested using collagenase as previously described (Cornish J, Callon KE, Lin CQX, Xiao CL, Mulvey TB, Cooper GJS, Reid IR Trifluoroacetate, a contaminant in purified proteins, inhibits proliferation of osteoblasts and chondrocytes. *Amer J Physiol Endocrinol Metab* 277: E779-E783, 1999). Primary rat osteoblasts were grown in DMEM supplemented with 10% FCS, 50 U/ml penicillin and 50 μ g/ml streptomycin. After 48 hour, the medium was changed to MEM. Confluence was reached within 5-6 days, at which time the cells were subcultured into 10cm culture plates for RNA preparation or 24 well plates for proliferation assays.

Preparation of mRNA

Total RNA was extracted from adult rat brain, skin, UMR106.06, or primary rat osteoblast cells using the guanidinium thiocyanate method (Chirgwin, 1979 #129). Poly (A)⁺ mRNA was purified from the total RNA using the PolyAtract mRNA Isolation System (Promega, Madison, WI).

Northern Blot Analysis

Primary rat osteoblast poly (A)⁺ (5µg) and rat brain poly (A)⁺ were size separated alongside lambda EcoRI/HindIII markers by electrophoresis on a 2.2M formaldehyde-1.2% agarose gel, transferred to a Magnacharge
5 Nylon membrane (MSI, Westborough, MA), and hybridised with a rat specific MC4-R gene DNA fragment spanning transmembrane domains III and VII (Mountjoy, 1994 #656). Hybridisation conditions were 50% formamide, 1mM NaCl, 50mM Tris-HCl (pH 7.5), sodium pyrophosphate (0.1%), SDS (0.2%), salmon sperm DNA (100µg/ml), 10x Denhardt's and
10 10% dextran sulfate at 42°C for 18h. A digital image of MC4-R transcripts was obtained after 10 days exposure with a phosphoscreen by using the Storm Imaging system scanner (Molecular Dynamics).

PCR amplification of reverse transcribed mRNA (RT-PCR)

Poly (A)⁺ mRNA was DNase treated twice using 10 U RQ1 RNase-free DNase (Promega Corp., Madison, WI) per mg poly (A)⁺ mRNA for 30
15 min at 37°C each time. First strand cDNA was synthesised using 200 U SuperScript II RNaseH⁻ reverse transcriptase (GIBCO BRL, Rockville, MD) and oligo (dT)₁₂₋₁₈ (Pharmacia Biotech AB, Uppsala, Sweden) at 42°C for 1h in a final volume of 20 µl. To test for DNA contamination of the RNA, a
20 reaction was carried out with 1 µg poly (A)⁺ mRNA and all the reagents but no reverse transcriptase (control reaction). The cDNA and control reaction (2 µl) were used as templates for PCR with rat melanocortin receptor specific oligonucleotides described in Table 1. The PCR conditions were
94°C for 3 min, 40 cycles of 94°C for 40 sec, annealing for 40 sec, and
25 72°C for 1 min, followed by 72°C for 10 min. The amplified cDNA products were separated on a 1.2% agarose gel alongside a EcoRI-HindIII-digested lambda DNA ladder and stained with ethidium bromide.

Ribonuclease protection assay

30 The cDNA templates used to synthesise the antisense rMC4-R and rMC1-R riboprobes were generated from 582 and 270 bp respectively, nucleotide DNA fragment spanning transmembrane I to VII and III to VI

domains subcloned into pBKS (Stratagene). These recombinant DNA templates were linearised with EcoRI and SalI transcribed with [α - 32 P]UTP (Amersham Life Science (Buckinghamshire, U.K.) using T7 RNA polymerase to generate 32 P-labeled cRNA probes. Rat brain or skin, UMR106.06, and primary rat osteoblast poly (A)⁺ mRNA (10 μ g) were treated with 2 U RNase-free DNaseI (Boehringer Mannheim, Indianapolis, IN) at 37°C for 50 min and the RNA was precipitated. The RNA pellet was resuspended in 20 μ l hybridization buffer (80% formamide, 40 mM PIPES pH 6.4, 400 mM NaCl, 1mM EDTA) with 5 x 10⁵ cpm of 32 P-labeled riboprobe, denatured at 85°C for 5 min and hybridized at 45°C overnight. The hybridised RNA was digested with 40 μ g RNase A and 50U RNase T1 at 37°C for 30 min. The protected RNA fragments were analyzed on a 6% denaturing polyacrylamide gel alongside a 32 P-labeled 123-bp DNA ladder (10⁵ cpm). A digital image of 32 P-labeled fragments was obtained using a Storm imaging system.

In Situ Hybridisation

Neonatal mouse calvariae, tibial and femoral bone were collected from 1-2 and 6 day old Swiss mice that had been euthanised by cervical dislocation while under halothane anesthesia (approved by the Auckland Animal Ethics Committee). The bones were dissected free of adherent soft tissues and fixed in 4% paraformaldehyde for 24h at 4°C prior to decalcification (15% EDTA, 4% paraformaldehyde) for 72 h at 4°C. They were then transferred to 10% sucrose, 4% paraformaldehyde overnight at 4°C before being embedded in OCT and stored frozen at -80°C. Five series of 20 μ M of either cross sectional or longitudinal sections were cut on the cryostat and mounted onto polysine coated microscope slides (Biolab Scientific, NZ) and in situ hybridisation performed as previously described (Mountjoy KG, Mortrud MT, Low MJ, Simerly RB, Cone RD Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain. Mol Endocrinol 8: 1298-1308, 1994). Bone sections were hybridised with 33 P labelled cRNA antisense rat MC4-R (628bp). Sections were hybridised in 65% formamide in 0.26M NaCl, 1.3x

Denhardt's, 13mM Tris-HCl pH 8.0, 1.3mMEDTA, 13% dextran sulphate at 60-65°C for 18 hours. Sections were washed and coated with emulsion for autoradiography. Following the developing of these slides, the sections were stained with haematoxylin and eosin and then photographed under darkfield on a Leica Microscope (Leitz DMRBE). One series of sections from each case was not subjected to In situ hybridization but was counterstained with haematoxylin and eosin and used for the identification of structures and bone cell type.

10 ***Primary rat osteoblasts proliferation assays***

Primary rat osteoblasts were subcultured into 24 well plates at a density of 5×10^4 cells/ml/well in MEM, 5% FCS for 24 hours. Cells were growth arrested in MEM, 0.1% bovine serum albumin (BSA) for 24 hour and then fresh media and experimental compounds were added for a further 24 hours. Cells were pulsed with [3 H]thymidine (0.5 μ Ci/well) 2 hours before the end of the experimental incubation. The experiment was terminated and both cell numbers and thymidine incorporation were assessed. Cell numbers were analysed by detaching cells from the wells by exposure to trypsin/EDTA (.05%/0.53mM) for approximately 5 minutes at 37°C. Counting was performed in a hemocytometer chamber. Results are expressed per well. [3 H]Thymidine incorporation was analysed by washing the cells in MEM followed by the addition of 10% trichloroacetic acid. The precipitate was washed twice with ethanol:ether (3:1) and the wells desiccated at room temperature. The residue was dissolved in 2M KOH at 55°C for 30 minutes, neutralized with 1M HCl, and an aliquot counted for radioactivity. Results are expressed as dpm per well. Each experiment was performed at least three times using experimental groups consisting of at least six wells.

30 ***Statistics***

Data are presented as mean \pm SEM. The significance of differences between groups was determined using Student's t tests for unpaired data and a

5% significance level. The comparisons to be made in each experiment were specified a priori, so no adjustment for multiple comparisons was necessary. Where several experiments have been shown in one figure, the data are expressed as the ratio of results in treatment groups to those in the control group and the 'P' values shown were calculated using the data from the individual experiments, before the data were pooled.

RESULTS

MC4-R mRNA is expressed in UMR106.06 and primary rat osteoblast cells.

Four different methods confirmed expression of MC4-R mRNA in UMR106.06 and rat primary osteoblast cells. First, RT-PCR, using rat specific MC4-R oligonucleotides amplified the correct size DNA fragment from poly A⁺ mRNA and not from genomic DNA. Second, Northern blot analysis of rat primary osteoblast poly (A⁺) mRNA (5 µg) showed a broad band of MC4-R mRNA transcripts between 2.0 and 2.6 kb, the same size as seen in rat brain, albeit of much lower abundance than in brain. Third, RPA's confirmed MC4-R mRNA expression in UMR106.06 and primary rat osteoblast cells. Finally, we used *in situ* hybridisation to localise MC4-R mRNA expression in the periosteum of 1-2 and 6 day old Swiss mouse calvariae, tibia, and femoral bones.

MC2-R and MC5-R mRNA are expressed in UMR106.06 and rat primary osteoblast cells.

RT-PCR, using rat specific MC2-R and MC5-R oligonucleotides amplified correct size DNA fragments from 1 µg UMR106.06 and 1 µg primary rat osteoblast cell poly A⁺ mRNA, but not from genomic DNA.

Alpha-MSH, but not desacetyl-α-MSH nor ACTH₁₋₂₄, stimulates proliferation of primary rat osteoblasts.

Alpha-MSH (10⁻⁹ – 10⁻⁷ M) significantly increased thymidine incorporation into growth arrested primary rat osteoblasts. Over a similar range of concentrations alpha-MSH also increased osteoblasts cell numbers. Desacetyl-α-MSH (10⁻⁷ M) and ACTH₁₋₂₄ (10⁻⁷ M) did not stimulate thymidine incorporation or cells numbers in growth arrested rat primary osteoblasts.

Desacetyl- α -MSH and ACTH₁₋₂₄ antagonise α -MSH stimulated proliferation of primary rat osteoblasts.

Desacetyl- α -MSH (10^{-7} M) inhibited two doses of α -MSH (10^{-8} M and 10^{-7} M) from stimulating [3 H] thymidine uptake into growth arrested rat primary osteoblasts (Figure 6a). ACTH₁₋₂₄ (10^{-7} M) inhibited two doses of α -MSH (10^{-8} M and 10^{-7} M) from stimulating [3 H] thymidine uptake into growth arrested rat primary osteoblasts.

Discussion

The MC4-R is likely to play a direct role in bone metabolism since its mRNA is expressed in a rat osteosarcoma cell line as well as in primary rat osteoblasts. The full length mRNA transcript for MC4-R expressed in primary rat osteoblasts is between 2 and 2.6 kb, the correct size for producing a functional protein in these cells. Expression of MC4-R mRNA is, however, much less abundant in osteoblasts than in rat brain, where MC4-R mRNA expression is already considered to be very low compared with many other genes. The MC4-R is not the only melanocortin receptor expressed in osteoblasts since we also observed MC2-R and MC5-R mRNA expressed in very low abundance in primary rat osteoblasts.

Despite the low abundance of melanocortin receptors, melanocortin peptides have significant biological effects on osteoblast cell proliferation.

Alpha-MSH (10^{-9} – 10^{-7} M) significantly stimulated both thymidine uptake and increased cell number in primary rat osteoblasts. The EC₅₀'s for α -MSH coupling mouse MC4-R and MC5-R to adenylyl cyclase or mobilisation of intracellular calcium are in the 10^{-8} M range, and therefore the α -MSH-stimulated osteoblast proliferation could be mediated by either MC4-R or MC5-R, or both. Alpha-MSH does not stimulate the MC2-R. Surprisingly, ACTH₁₋₂₄ had no significant effect on osteoblast proliferation and yet ACTH₁₋₂₄ functionally couples MC2-R, MC4-R, and MC5-R to adenylyl cyclase when these receptors are overexpressed in various cell lines. Desacetyl- α -MSH (10^{-7} M and 10^{-8} M) also had no significant effect on osteoblast proliferation in two out of three experiments, and yet the EC₅₀'s

for desacetyl- α -MSH coupling MC4-R and MC5-R to intracellular signaling pathways when these receptors are overexpressed in heterologous cells are similar to those for α -MSH.

To further understand the significance of MC4-R mRNA expression
5 in osteoblasts we attempted to antagonise the α -MSH stimulated osteoblast proliferation. Agouti protein is an antagonist of melanocortin peptides coupling MC1-R, MC2-R, and MC4-R. However, in our study agouti protein alone (10^{-9} M– 10^{-7} M) significantly stimulated thymidine incorporation in primary rat osteoblasts and did not antagonise α -MSH stimulated
10 osteoblast proliferation. Furthermore, agouti protein stimulated-thymidine incorporation was not additive with α -MSH stimulated-thymidine incorporation, suggesting that agouti protein and α -MSH may be having their effects through the same melanocortin receptor and signal transduction pathway.

We were unable to distinguish between the three subtypes of
15 melanocortin receptors expressed in osteoblasts based on biological activities of melanocortin receptor agonists, and the MC2-R/MC4-R antagonist, agouti protein. This is not the first time however, that the biological activities of melanocortin receptor ligands on endogenous
20 melanocortin receptors differ from their biological potencies on cloned melanocortin receptors overexpressed in heterologous cells. First, α -MSH and desacetyl- α -MSH are potent agonists of the cloned MC1-R overexpressed in heterologous cell lines, but only α -MSH potently stimulates pigmentation in rodent skin. Second, NDP-MSH is a potent
25 agonist of cloned MC5-R overexpressed in heterologous cell lines, but it is a potent antagonist of α -MSH activation of adenylyl cyclase in 3T3-L1 adipocytes. It is possible that the very low expression of endogenous melanocortin receptors in primary osteoblasts, melanocytes, and 3T3-L1 adipocytes contributes to the differences in melanocortin potencies in these
30 cells compared with overexpressed cloned melanocortin receptors. Additionally, 3T3-L1 adipocytes, like primary osteoblasts, express more than one melanocortin receptor subtype. It is likely therefore, that

heterodimeric receptors are formed and these could have different pharmacological profiles from homodimers formed when each cloned melanocortin receptor is overexpressed alone.

Without wishing to be bound by any particular mechanism of action it is proposed that osteoblasts are a model system for understanding interactions between melanocortin receptor ligands and melanocortin receptors, and this model system more closely resembles *in vivo* responses to melanocortin peptides compared with overexpressing only one melanocortin receptor in an heterologous cell. It has been shown that while desacetyl- α -MSH or ACTH₁₋₂₄ alone had no agonist effects on osteoblast proliferation, they were both capable of antagonising α -MSH stimulated osteoblast proliferation. This study is the first to report the ACTH₁₋₂₄ antagonism of α -MSH. Desacetyl- α -MSH antagonises α -MSH stimulated mammatrope recruiting activity in anterior pituitary cell cultures (Ellerkmann E, Kineman RD, Porter TE, Frawley LS Des-acetylated variants of α -melanocyte -stimulating hormone and β -endorphin can antagonize the mammatrope-recruiting activity of their acetylated forms. J Endocrinology 139: 295-300, 1993) and antagonises α -MSH activity on *Anolis* melanophore (McCormack AM, Carter RJ, thody AJ, Shuster S Des-acetyl MSH and γ -MSH act as partial agonists to α -MSH on the *Anolis* melanophore. Peptides 3:13-16, 1981).

Low level endogenous expression of three melanocortin receptor subtypes in osteoblast cells provides a model system (Figure 8) for exploring interactions between melanocortin receptor ligands and melanocortin receptors that will more accurately reflect the *in vivo* actions of melanocortin peptides, agouti, and agouti gene related peptide. In osteoblasts, and probably many cell types expressing low levels of endogenous melanocortin receptors, there is the likelihood of melanocortin receptor homo- and heterodimers, and cross talk between different melanocortin receptors. These interactions would provide diversity and specificity for melanocortin peptide signalling that would not be available

when a single melanocortin receptor subtype is overexpressed in heterologous cells.

It is evident that a variety of cell types and tissues may express melanocortin receptors. In addition to those described above, any such cells or tissues would be appropriate candidates as a biological response system, according to the invention described herein. Examples of cell lines that could be utilised in a similar manner as described above include the GT1-7 mouse hypothalamic cell line, 3T3-L1 adipocytes, melanocytes, L6 myocytes, B16 melanoma cells, and anterior pituitary cell cultures.

- Genetically engineered, or heterologous cell lines that stably express a single or a combination of melanocortin peptides are also good candidates as *in vitro* cellular biological response systems. A panel of such cell lines, each expressing a different melanocortin receptor may comprise a biological response system. Alternatively, co-cultures of two or more heterologous cell lines, each expressing different melanocortin receptors may comprise a biological response system.

Example 6: Biological response by UMR106.06 rat osteosarcoma cell line.

20 Incorporation of tritiated thymidine into DNA

- UMR106 cells are plated at at 1×10^5 cells/well in a 24 well plate using 10% FCS, DMEM media. 24 hours later the medium is changed to serum free medium containing 0.1% BSA. Following a 24 hour incubation period, the medium is changed again to serum free media containing 0.1% BSA and increasing concentrations of melanocortin peptides. The cells are then incubated for 22 hours. Following this period of incubation {methyl- ^3H } thymidine [0.5 μCi in 25 μl /well] is added and left for 2 hours at 37°C (use 0.5 μl of 1 $\mu\text{Ci}/\mu\text{l}$ tritiated thymidine into 24.5 μl 0.1% BSA, DMEM for each well). The experiment is terminated by washing the cells with 1ml cold PBS and then add 1ml cold 5% TCA.

The plates are then left at 4°C (on ice) for 15 minutes and then washed 3 x with 1ml cold 5% TCA and twice with 1ml absolute ethanol. The

monolayers are air dried and cells dissolved in 1ml 0.3N NaOH by heating at 37°C for 1 hour. 200µl of 1.5N HCL is then added to each well and then the contents of each well is transferred to individual 20ml glass scintillation vials. 7 mls of scintillation fluid is added and mixed well. The samples are

5 counted for 5 minutes.

RESULTS

Figures 9 and 10 show the proliferation response resulting from the treatment of UMR106.06 rat osteosarcoma cells with varying concentrations of alpha-MSH or desacetyl-alpha-MSH.

10 This example is illustrative of the usefulness of a permanent cell line that can be used as an *in vitro* biological response system. Of course, it will be understood that a proliferative response is only one of many response parameters that may be utilized as a response profile.

15 Example 7: Use of the *in vitro* biological response system to screen for compounds that act as agonists or antagonists of melanocortin receptors.

20 An *in vitro* biological response system may be utilised to screen for compounds that act as agonists or antagonists of melanocortin receptors. Such a biological response system could also be utilised to screen for compounds that are useful in the treatment of subjects suffering from obesity or an imbalance in energy homeostasis or disturbance in feeding/weight gain patterns.

25 The screening process involves treating the cells of the biological response system having the appropriate combination of receptors with test compounds and then measuring the response parameters, either by mass spectrometry or by gene expression array or by other available techniques which are able to assess the required response parameters. The
30 compound that produces the desired response profile is a compound which may be useful in the treatment of obesity or imbalances in energy homeostasis and/or disturbances in feeding/weight gain patterns. The

biological response system will also enable the selection of compounds that are able to block the undesirable effects of environmental and nutritional factors that cause obesity or imbalances in energy homeostasis and/or disturbances in feeding/weight gain patterns.

- 5 The profile generated by compounds that produce a desired response in an *in vitro* biological response system may then be compared with the profile that is generated from the administration of the compound to an *in vivo* biological response system.

10 **Example 8: *In vitro* biological response of 3T3 L1 Adipocytes to melanocortin peptides.**

(i) **Culturing Murine 3T3 L1 Cells**

Culturing and passaging cells based on methods described in references

- 15 Norman D et al Mol Cell Endocrinol 200: 99-109, 2003; Hasegawa N et al Phytother Res 16: S91-S92, 2002; Student AK et al J Biol Chem. 255: 4745-4750, 1980; and Ross SE et al Mol Cell Biology 19: 8433-8441, 1999, all of which are incorporated herein in their entirety by reference.

Reagents

20 1.1 **Growth Medium:**

a-MEM culture medium: powder from GibcoBRL, prepared in advance and stored in volumes of 225 ml in sterile culture bottle at 2-8°C.

- 25 ***Fetal Bovine Serum (FBS):*** GibcoBRL, sterile heat-inactivated serum stored in 25 ml aliquots in 50 ml tubes in -20°C freezer. To heat inactivate place serum in water bath set to 50°C for 1 hour.

Penicillin/Streptomycin (P/S): (GibcoBRL 15070-063, 100 U/ml, 100 mg/ml,) stored in sterile aliquots in -20°C freezer.

- 30 1.2 **Reagents for passaging pre-adipocytes**
Growth medium

Trypsin: (GibcoBRL 25300-024, 100 ml) stored in sterile 15 ml tube aliquots in -20°C freezer.

Versene: (GibcoBRL 15040-066, 1:5000, 100 ml) stored in sterile bottle at 2-8°C. Versene is EDTA, a calcium chelator used to remove calcium, which helps cells attach to plate.

5

4.1 **Plating cells:**

Transfer cell suspension from cryotube to a 5ml medium tube, centrifuge at room temperature (20-22°C) at 960 rpm for 5min, aspirate supernatant, leaving approximately 2mm supernatant above pellet so that cell pellet is not disturbed.

10

Add 10 ml medium and resuspend with 10 ml pipette, gently drawing up medium and releasing along side of tube approximately 10 times to disperse cells.

Transfer cell suspension to labelled petri dish (tech name, date, cell ID) and examine under microscope (10x objective), checking that there are no cell clumps. Place in incubator at 37°C and 5% CO₂.

15

Passaging Pre-adipocytes to Increase Cell Number

20 **Detaching cells from plate:**

5.1 Pre-adipocytes are ready for passaging every 4-5 days (cells are not confluent and generally only 5-10% differentiated).

- Transfer 5 ml growth medium into 15 ml tube.
- Remove culture plate of 3T3 L1 cells from incubator and place in hood. Aspirate medium.
- Add 2 ml Versene to plate, allowing it to run down inside wall of plate to avoid dislodging cells. Gently swirl to run over whole bottom of plate, then aspirate immediately.
- Add 2 ml trypsin over whole bottom of plate. Tap bottom of plate, place in incubator for ~1 minute, check under microscope that cells are dislodged and not clumpy.

25

30

- Transfer cells to tube with 5 ml medium and centrifuge at approximately 21°C for 5 minutes at 960 rpm.

5.2 *Passaging cells :*

- 5 • While cells are spinning, place 9 ml fresh medium into each labelled culture plate.
- After spin, aspirate cell supernatant (down to ~1 mm from pellet).
- 10 • Add 10 ml medium and mix to resuspend with several up/down strokes (~10).
- Transfer 1 ml into each plate.
- Examine under microscope to check cells and for absence of cell clumps.
- Place in incubator, 37°C and 5% CO₂.
- 15 • Discard remainder of cells in sealed tube in biohazard bag.

Oil Red O Staining of Adipocytes

- Oil Red O staining is used to determine differentiation efficiency of adipocyte cell lines such as 3T3 L1 cells by staining intracytoplasmic lipid accumulation. This method is broadly based on methods published earlier (Norman D et al Mol Cell Endocrinol 200: 99-109, 2003; Ross SE et al Mol Cell Biology 19: 8433-8441, 1999; Zhang HH et al J Endocrinol 164: 119-128, 2000, incorporated herein in their entirety by reference).

25

Materials and Preparation of Reagents

Isopropanol

- 100% isopropanol
- 60% isopropanol = 60 mL isopropanol + 40 mL distilled H₂O
- 30 50% isopropanol = 50 mL isopropanol + 50 mL distilled H₂O

Oil Red O Stain

Use at 0.3% in 60% isopropanol

0.3% stain = 300 mg Oil Red O + 100 mL 60% isopropanol

Filter before use.

Phosphate Buffered Saline (PBS) sterile for cell culture, pH 7.4

8 g NaCl + 0.2 g KCl + 1.44 g Na₂HPO₄ + 0.24 g KH₂PO₄.

5

Dissolve in ~800 ml milli Q water. Adjust pH to 7.4 with 1N HCl.

Bring volume up to 1 L and autoclave.

4% paraformaldehyde, pH 7.4

4% = 4 g paraformaldehyde + 100 mL PBS

10

Dissolve by adding 1 pellet NaOH while mixing on heated mixer (~50°C).

Adjust pH to 7.4 with 1N HCl.

Staining Cells

15

Use the same volume for each reagent, which is determined by plate/well size as Table 1.

Aspirate cell medium and rinse 2x with PBS.

Fix for 1 hour in 4% paraformaldehyde at 4°C (place in fridge or cold room).

20

Aspirate paraformaldehyde and rinse 2x with PBS.

Stain with Oil Red O for 20 minutes, leave plate in hood.

Aspirate stain and rinse 2x with water and 1x with 50% isopropanol.

Check staining of cells under microscope.

25

Elute stained lipids with 100% isopropanol. Check elution efficiency under microscope.

Measure absorbance at 510 nm on spectrophotometer.

Table 1 Volume of Reagents Used for Oil Red O Staining

30

Plate	Diameter of well/plate	Area of well/plate	Volume of Reagents
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	(mm)	(mm ²)	(mL)
12-well dish	20	314	0.5
6-well dish	35	962	2
Culture plate	100	7854	10

Differentiation of 3T3 L1 Cells with Indomethacin

5 Growing Cells and Inducing Differentiation

Differentiation induction with indomethacin based on Norman et al (Norman D et al Mol Cell Endocrinol 200: 99-109, 2003). Details of preparation of α MEM growth medium (containing 10% FBS and pen/strep), retrieving and plating cryopreserved 3T3 L1 cells from liquid nitrogen are detailed above.

Passage cells when nearly confluent, in 4-5 days, by splitting 1/10 in new plates and feed every 2 days.

To induce differentiation, 48 hours after cells are confluent add differentiation medium as follows (DAY 0):

15 Prepare differentiation medium as in 2.0.

Aspirate growth medium from plate.

Add differentiation medium to plate and return to incubator, 37°C and 5% CO₂. Volume depends on size of well or plate. Use 2 ml/well in 6-well plate or 10 ml/culture plate.

20 After 48 hours differentiation (DAY 2), aspirate differentiation medium and add growth medium supplemented with 5 ug/mL Insulin. Change medium every 2 days.

Perform experiments on DAY 12-14, or later if desired.

Preparation of Indomethacin Differentiation Medium

25

Indomethacin: (Sigma I 7378, MW = 357.8). Use at a final concentration of 125 uM. On day of use dissolve 15 mg/ml in

45

absolute ethanol. A final concentration of 125 μ M indomethacin requires 44.725 μ g/ml growth medium or 4472.5 μ g/100 ml.

$$1 \text{ M} = 357.8 \text{ g/L} = 357.8 \text{ mg/ml}$$

$$1 \text{ mM} = 357.8 \text{ }\mu\text{g/ml}$$

$$1 \text{ }\mu\text{M} = 0.3578 \text{ }\mu\text{g/ml}$$

$$125 \text{ }\mu\text{M} = 0.3578 \times 125 = 44.725 \text{ }\mu\text{g/ml}$$

For 100 ml medium, use $100 \times 0.044725 \text{ mg/ml} = 4.47 \text{ mg}$.

$$4.47 \text{ mg} = 298 \text{ }\mu\text{l of } 15 \text{ mg/ml solution } (4.4725/15 = 298 \text{ }\mu\text{l}).$$

Insulin (bovine): (Sigma I 6563, MW = 5733.5). Use at a final concentration of 5 μ g/ml. Prepare a 1 mg/ml solution (store unused solution at -20°C). For 100 ml medium, use $100 \times 0.005 \text{ mg/ml} = 0.5 \text{ mg}$, which is 0.5 ml of 1 mg/ml.

Calculate volume of differentiation medium required (as in 1.3.3). For 100 ml growth medium add:

- 298 μ l of 15 mg/ml indomethacin solution
- 500 μ l of 1 mg/ml insulin solution.

Mix by swirling.

Stimulation of 3T3 L1 Adipocytes with Melanocortin Peptides

25 1.0 Methodology:

Method according to Norman D et al (2003) Mol Cell Endocrinol 200, p 99-109 was used. This publication is incorporated in its entirety herein by reference.

30 2.0 Introduction and Overview

- 2.1 The objective of this study was to determine the effects of α -MSH and desacetyl α -MSH on leptin and triglyceride production in murine 3T3 L1 adipocytes.
- 2.2 Pre-adipocytes were seeded in 6-well plates and 2 days post confluence (Day 2) were differentiated with 125 mM indomethacin + 5 ug/mL insulin (described in previous documents).
- 2.3 On Day 13 adipocytes were stimulated with 4 doses each of α -MSH and desacetyl α -MSH (or no peptide added) for 4 hours.
- 2.4 Medium was removed from the wells and leptin and triglyceride levels measured.
- 2.5 Intracytoplasmic lipid accumulation was measured by staining with Oil red O.

3.0 Reagents

- 3.1 α -MEM growth medium
- 3.2 Bovine Serum Albumin (BSA)
- 3.3 α -Melanocortin Stimulating Hormone (α -MSH), MW 1665
- 3.4 desacetyl α -Melanocortin Stimulating Hormone ($d\alpha$ -MSH), MW 1623
- 3.5 Phosphate Buffered Saline (PBS), pH 7.4
- 3.6 Isobutylmethylxanthine (IBMX) Sigma I 7378, MW = 222.2.

4.0 Preparation of Reagents

- 4.1 Medium = α -MEM + 0.5% BSA (100 mL α -MEM + 0.5 g BSA)
- 4.2 Doses of α -MSH and $d\alpha$ -MSH (stocks in -80°C freezer = 1 ug/ul), using MW of α -MSH.
- 1 M = 1665 g/L = 1665 mg/mL
- 1 mM = 1.665 mg/mL

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1 μM = 1.665 $\mu\text{g/mL}$

1.665 $\mu\text{g/mL}$ = 3.3 $\mu\text{g/2 mL}$ in each well

1/10 dilution of 1 $\mu\text{g/ul}$ (stock) = 0.1 $\mu\text{g/ul}$. 33 μl = 3.3 μg .

- Prepare 1/10 dilution of freezer stock (1 $\mu\text{g/ul}$) to make 0.1 $\mu\text{g/ul}$, using α -MEM + 0.5% BSA as diluent.
- Make 3 serial dilutions of 1/10 to add 33 μl to each well in 6-well plate.
- Doses are in triplicate wells, so require $3 \times 33 = 99 \mu\text{l}$ for each dose.

Final Dose when adding 33 $\mu\text{l/well}$	Stock	Dilution
1 μM	A	20 μl freezer stock + 180 μl medium
100 nM	B	20 μl stock A + 180 μl medium
10 nM	C	20 μl stock B + 180 μl medium
1 nM	D	20 μl stock C + 180 μl medium

4.3 1 mM IBMX (final concentration) = 0.2222 mg/mL.

Immediately prior to use on Day 13, prepare 30 mg/6 mL solution in sterile PBS as in "Differentiation with Dexamethasone and IBMX" document. 100 mL medium requires 22.22 mg, which is 4.44 mL of solution ($22.22/30 \times 6 \text{ mL} = 4.44 \text{ mL}$).

5.0 Peptide Stimulation Assay

5.1 Assay is performed on Day 14 after Initiation of cell differentiation. On day prior to stimulation assay, replace growth

medium + insulin with medium prepared in 4.1 (α -MEM + 0.5% BSA) and return plates to incubator.

5.2 On Day of assay prepare peptide solutions as in 4.2 and IBMX as in 4.3.

5.3 Replace medium with the same medium supplemented with 1 mM IBMX (as in 4.3) and allow cells to equilibrate in incubator for 10 minutes.

5.4 Add increasing concentrations of peptides (or none), 33 μ l per well, swirl gently to mix, and place plates in incubator for 4 hours.

5.5 At the end of the incubation remove media from wells and store triplicate aliquots in -20°C freezer for measurement of leptin and triglycerides.

5.6 Stain adipocytes in wells with Oil Red O as in "Oil Red O Staining of Adipocyte" document.

5.7 After eluting the stain, remove cells from wells as in 6.0 for measuring total protein.

Table 5: Effect of α -MSH or desacetyl- α -MSH on leptin production in differentiated 3T3L1 adipocytes.

Leptin results are from 2 separate 4-hour peptide stimulation assays of triplicate incubation wells for each dose. For each assay, leptin was measured in duplicate samples from triplicate incubation wells and data was normalised to percentage of control (results with no added peptide). Mean control leptin results for the 2 assays were 930 ± 47 pg/mL and $535 \pm$

61 pg/mL. Data in the table is the combined normalised results from the 2 assays, showing mean % of control \pm SEM.

PEPTIDE DOSE	Leptin Response with α MSH			Leptin Response with desacetyl α MSH		
	Mean	SEM	n	Mean	SEM	n
0 (Control)	100.0		6	100.0		6
1 nM	100.8	4.5	11	95.4	2.3	11
10 nM	107.4	6.4	10	96.3	3.6	12
100 nM	100.9	5.7	9	97.7	5.0	12
1000 nM	98.0	3.3	12	109.4	6.5	12

- 5 There is a trend for desacetyl- α -MSH but not α -MSH to reduce leptin production over this time period. The triglyceride levels did not appear to change (see Table 6) and therefore this reduction in leptin production may reflect a reduction in leptin gene transcription.

Leptin Assays: Quantikine M kit (R & D Systems Inc, UK # MOB00

- 10 Abingdon, Oxon) and DSL kit (DSL #10-24100, Australia PTY Ltd, NSW, Australia) were used. Both are specific for murine leptin, validated for use with cell culture medium, and showed a similar result for an in-house quality control pooled murine plasma sample. The Quantikine M kit is preferred as it is more sensitive and precise.

15

Table 6: Effect of α -MSH or desacetyl- α -MSH on triglyceride release from 3T3 L1 adipocytes in the 2 peptide stimulation assays in Table 5.

- 20 Results in each peptide stimulation assay were normalised to percentage of control. Data in the table is the combined normalised results from the 2 assays, showing mean % of control \pm SEM.

PEPTIDE ADDED	PEPTIDE DOSE	TRIGLYCERIDE RESPONSE (% OF CONTROL)	
		MEAN	SEM
NONE (CONTROL)	0	100	
AMSH	100 nM	110.5	5.6
	1000 nM	105.7	9.3
DA MSH	100 nM	111.4	6.7
	1000 nM	110.0	7.2

Table 7: Effect of different ratios of alpha-MSH and desacetyl-alpha-MSH on leptin production in differentiated 3T3L1 cells.

In one of the two peptide stimulation assays described in Table 5, 7
 5 different peptide ratios (as indicated in the table below) were added to
 triplicate wells. Leptin was measured in duplicate samples from each well.
 Data shown is mean leptin level \pm SEM (pg/mL) from the single dose
 concentrations of each peptide and the 3 ratios.

Peptide	Concentration	Mean	SEM	n
aMSH	1n M	544.2	54.6	5
	100 nM	547.0	52.9	6
desacetyl aMSH (da MSH)	1n M	480.4	14.5	5
	100 nM	528.0	53.9	6
1 nM aMSH + 100 nM da MSH		417.8	68.0	5
100 nM aMSH = 1 nM da MSH		562.3	68.5	6
1 nM aMSH + 1 nM da MSH		575.0	29.2	6

Compared to 1 nM alpha-MSH and 100 nM desacetyl-alpha-MSH, the ratio of 100 nM desacetyl-alpha-MSH/ 1 nM alpha-MSH appears to reduce leptin production. Therefore an abundance of desacetyl-alpha-MSH may lead to reduced leptin gene transcription.

5 While 1 nM desacetyl-alpha-MSH appears to reduce leptin production, the ratio of 1 nM desacetyl-alpha-MSH/100 nM alpha-MSH does not appear to reduce leptin production and neither does 100 nM alpha-MSH alone. Therefore an abundance of alpha-MSH may prevent desacetyl-alpha-MSH from reducing leptin gene transcription.

10 While 1 nM desacetyl-alpha-MSH appears to reduce leptin production, the ratio of 1 nM desacetyl-alpha-MSH/1 nM alpha-MSH does not appear to reduce leptin production and neither does 1 nM alpha-MSH alone. Therefore an equimolar concentration of alpha-MSH may be sufficient to prevent desacetyl-alpha-MSH from reducing leptin gene
15 transcription.

 It will be understood from the foregoing that either a reduction in the level of alpha-MSH or the increase in the level of desacetyl-alpha-MSH will result in a higher desacetyl-alpha-MSH : alpha-MSH ratio. Further, a reduction in the level of alpha-MSH or desacetyl-alpha-MSH individually,
20 with respect to sex and age matched reference ranges, may also be used effectively in the methods of the present invention. Not wishing to be bound by any particular theory, it is likely that desacetyl-alpha-MSH alone, at levels above a particular threshold, would be useful in the methods of the present invention.

25 Although the invention has been described with reference to specific examples, it will be appreciated by those skilled in the art that the invention may be embodied in many other forms.